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Flow Cytometric Measurement of Rat Lymphocyte Subpopulations After Burn Injury and Burn Injury With Infection

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Increased infection rates in burned patients may result from a disproportionate increase in the suppressor subpopulations. Measurement of lymphocyte subpopulations is difficult in burned patients because gradient-purified cells are contaminated by nonlymphoid cells. The accuracy of flow cytometric subpopulation analysis was improved by restricting (gating) the analysis to cells with light-scatter intensity typical of lymphocytes. Blood was obtained 48 hours after burn from rats receiving no burns, 30% scald burns, or burns seeded with *Pseudomonas aeruginosa* to induce infection. Subpopulations were identified by monoclonal antibodies to T-lymphocyte antigens. Gating increased the values obtained for most subpopulations, but the relative differences between groups were unchanged. Burned and infected animals, but not animals burned only, had a decreased ratio of helper to suppressor lymphocytes (HSR) relative to control. A decreased HSR correlated with sepsis, but not with infection susceptibility. This suggests that a decrease in HSR may be a result of infection rather than a cause of susceptibility to infection.

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Hematologic cellular responses to burn trauma typically include leukocytosis, lymphopenia, and monocytosis.[1] The relationship between mortality due to infection and the changes in the cellular components of host defense is still a mystery. Many changes of in vitro immune cell function have been described in burned patients, but there is no conclusive evidence that these changes underlie defective host defense mechanisms.

Several studies have reported changes in the relative proportions of lymphocyte subpopulations in burned patients. The helper-suppressor ratio (HSR) has been reported to be decreased in burned patients up to 21 days after burn,[2] and a decreased HSR more than 14 days after injury was reported to be associated with an increased frequency of sepsis and mortality.[3] These observations suggest the possibility that increased proportions of suppressor cells may initiate the susceptibility to infection observed in burned patients.

An accurate evaluation of the role of lymphocyte function and phenotype in host defense requires that observations be made on samples of known cell composition. Studies of lymphocytes from burned patients are difficult because of contamination of lymphocyte preparations by other cell types.[4,5] The complex morphologic and physical changes in the blood leukocyte populations subsequent to burn injury result in co-purification of abnormal granulocytes and monocytes with the lymphocytes on Ficoll-Hypaque density gradient separation procedures. The problem is often exaggerated by lymphopenia. The interpretation of in vitro studies is uncertain because the extent of the contamination may not be known, and the effect of the contamination on the parameters being measured is unpredictable even if the extent of contamination is known. In this report we describe subpopulation analysis in a rat burn model in which density-purified leukocytes were preanalyzed by light scatter during flow cytometry to exclude contaminating leukocytes and increase the accuracy of the results.

MATERIALS AND METHODS

Male albino rats (300 to 400 g) were randomly assigned to one of three groups: an unburned control group, a burned group, and a burned group with infection. All animals were anesthetized with pentobarbital (1 mg/100 g of body weight, given intraperitoneally); those in the burned groups were shaved and subjected to a full-thickness burn covering 30% of total body surface area by 10-s immersion in boiling water. Infection was induced by seeding 1 mL of a 16-hour broth culture containing approximately 10^8 *Pseudomonas aeruginosa* (strain 59-1244) on the burned dorsum within one hour of scalding. The rats were anesthetized with pentobarbital 48 hours after burn and exsanguinated by drawing blood from the hepatic vein. A portion of the blood sample (1 mL) was cultured in trypticase soy broth to ascertain the presence of bacteria.

Total leukocyte counts were made with an automated counter, and blood smears were prepared for differential analysis. The cells having densities characteristic of normal lymphocytes were separated from other blood cells by centrifugation on a Ficoll-Hypaque density gradient. A portion of the isolated cells was used to prepare a slide for differential analysis, using a cytocentrifuge to assess the

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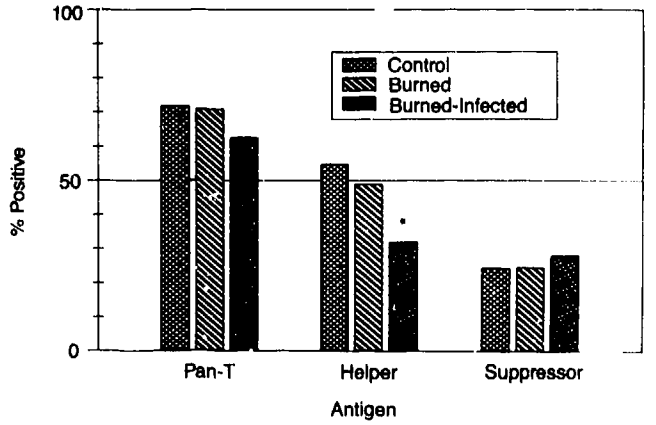

Fig 1.—Ficoll-Hypaque–purified leukocytes (ungated) positive for T-lymphocyte antigens. Cells from burned, burned-infected, and control rats were analyzed by flow cytometry for each of three surface antigens. Positive values are expressed as mean percentage ± SD of total cells analyzed. Asterisk indicates $P<.05$.

Table 1.—Helper-Suppressor Ratio Determined for Ficoll-Hypaque–Purified Rat Lymphocytes*

Group	Mean ± SD Ratio	P
Control	2.28 ± 0.30	. . .
Burned	2.13 ± 0.32	Not significant vs control
Burned-Infected	1.03 ± 0.54	<.001 vs control, <.001 vs burned

*Percentages of T-helper and T-suppressor cells were determined by flow cytometry and used to calculate the helper-suppressor ratio for each animal. The mean values and SDs were calculated for ten animals in each group.

Table 2.—Differential Analysis of Ficoll-Hypaque Preparations by Light Scatter and Wright's Stain Morphologic Findings*

	% Lymphocytes (Mean ± SD)	
Group	By Light Scatter	By Wright's Stain
Control	77.6 ± 11.01	91.0 ± 9.9
Burned	73.8 ± 8.8	87.0 ± 11.4
Burned-Infected	56.4 ± 16.3	74.9 ± 16.4

*Cells were analyzed for light-scatter characteristics by flow cytometry, and the proportion of cells falling within the lymphocyte "window" was determined. A small aliquot containing approximately 10^5 cells was used to make a cytocentrifuge slide that was stained with Wright's stain and examined for the relative number of lymphoid and nonlymphoid cells under light microscopy.

purity of the lymphocyte preparation. The remaining cells were stained with appropriate anti–rat lymphocyte monoclonal reagents, washed once, and further reacted with affinity-purified, fluorescein-labeled, goat anti–mouse IgG (Fab_2' fragments) as a second-step reagent.

The fluorescein-labeled cells were analyzed on a flow cytometer. For each sample, 5000 cells were analyzed, and the numbers of cells labeled by fluorescent monoclonal antibodies specific for the pan–T-lymphocyte (W3/13), helper-inducer (W3/25), or suppressor-cytotoxic (OX-8) cell surface markers were determined. A negative control using a monoclonal antibody to human T cells was run with each cell preparation to determine the cutoff that distinguished negative and positive cells. The positive cutoff was set at a point defining the upper 5% or less of the background control, and the number of background control cells beyond the cutoff was subtracted from each sample. For gated analysis, nonlymphoid cell

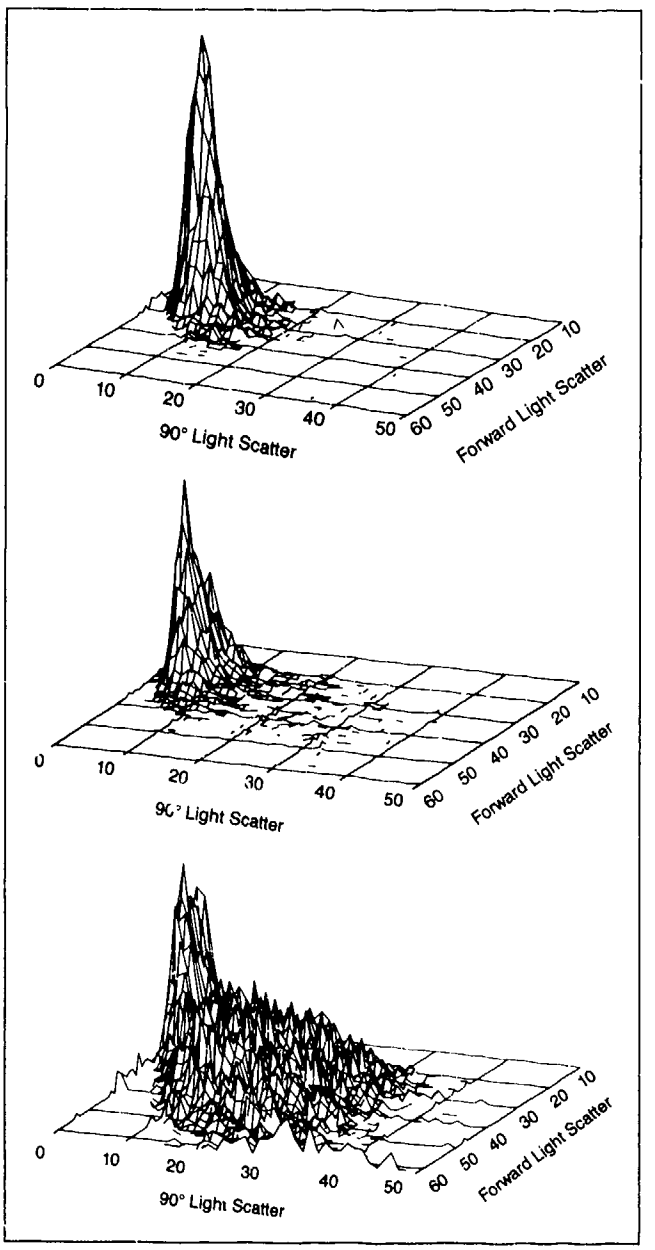

Fig 2.—Forward angle light scatter and 90° scatter histograms. Light-scatter intensities for 5000 Ficoll-Hypaque–purified cells determined by flow cytometry are displayed in two-parameter histograms. Top, Control; middle, burned; and bottom, burned-infected.

contamination was monitored by analyzing forward-angle light scatter (FALS) and 90° scattered light. Cells with scattered-light intensities that fell outside limits established for normal lymphocytes were removed from analysis (gated).

Statistical analysis consisted of a pairwise analysis of means (t test) with a Bonferroni adjustment, performed on a minicomputer. Results of gated vs ungated analyses were evaluated using Wilcoxon's matched pairs signed-ranks test.

RESULTS

Ficoll-Hypaque–purified peripheral-blood lymphocytes isolated from the animals in each group were analyzed for T-cell subpopulation surface markers. After ungated flow

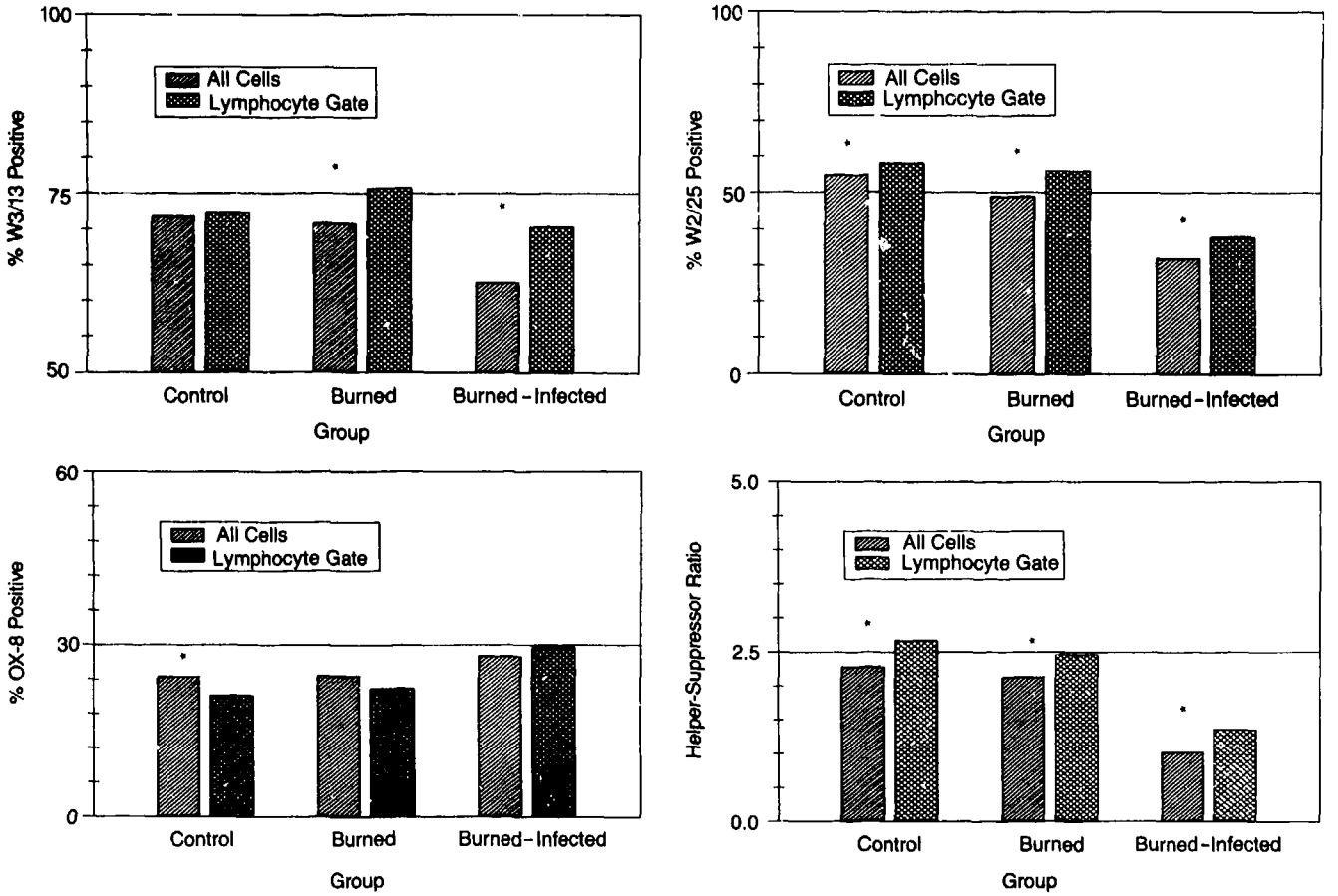

Fig 3.—Effect of light-scatter gating on T-lymphocyte populations. Mean percentage positive for each group was determined before and after gating. Top left, Pan-T antigen (W3/13); top right, helper-T antigen (W2/25); bottom left, suppressor-T antigen (OX-8); and bottom right, helper-suppressor ratio. Asterisk indicates $P<.01$ ($n=10$).

cytometric analysis, the number of lymphocytes from animals in the burned-infected group was drastically reduced from that of the other two groups (55% of control; $P<.01$). The percentages of cells positive for pan-T (W3/13), helper-inducer (W3/25), and suppressor-cytotoxic (OX-8) markers in burned animals were not significantly different from those of unburned control animals (Fig 1). The burned-infected animals, however, had a significantly reduced proportion of cells positive for W3/25 ($P<.05$). The HSR for this group was also significantly reduced (Table 1).

Analysis of light scatter from the cells obtained from animals of each group revealed significant differences in FALS and 90° scatter for animals in each group (Fig 2). Intensity of FALS is proportional to cell size, while 90° light-scatter intensity is related to the amount of cytoplasmic structure present in the cell (ie, granules and Golgi structure). Ficoll-Hypaque preparations from control animals were composed of 91.9% lymphocytes as judged by morphologic findings after Wright's stain (Table 2). In the isometric display in Fig 2, top, the Ficoll-Hypaque lymphocytes from a control animal were concentrated in one peak near the origin and the monocytes (which have greater intensity of FALS and 90° scatter than lymphocytes) were scattered beyond the lymphocyte peak further from the origin. Lymphocyte preparations from burned rats (Fig 2, middle) had an increased number of cells (mostly monocytes) with light-scatter intensity greater than that of normal lymphocytes. Preparations from burned-infected rats had a large proportion of cells with increased light-scatter intensity (Fig 2, bottom), which corresponded with an increase in the number of cells with nonlymphoid morphologic features seen by light microscopy. Most of these nonlymphoid cells had the morphologic characteristics of immature granulocytes, reticulocytes, agranular polymorphonuclear leukocytes, or monocytes.

Since the presence of nonlymphoid cells could distort the values obtained for the lymphocyte subpopulations, a more accurate value might be obtained if only those cells scattering light typical of lymphocytes were included in the analysis. The data were reanalyzed with a predetermined light-scatter window set to exclude electronically from analysis cells falling outside the limits of normal lymphocytes (a process referred to as gating). The relative number of cells falling within the lymphocyte gate was compared with the number of lymphocytes determined by light microscopy after Wright's stain (Table 2). Because light scatter is less specific than morphologic features on Wright's stain in differentiating lymphocytes from other cells, a highly restrictive light-scatter window is set to ensure that all nonlymphoid cells are excluded from the analysis. As a

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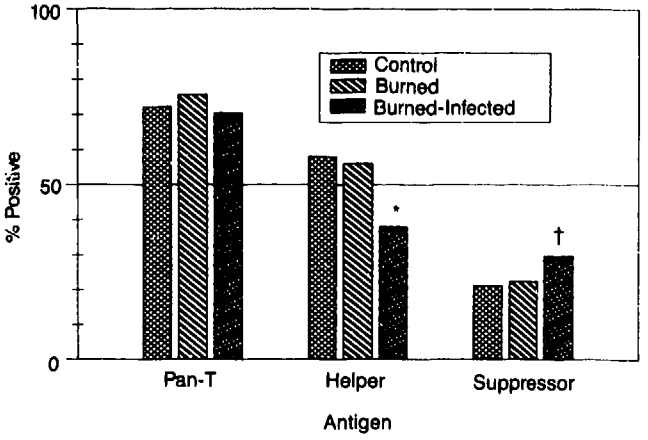

Fig 4.—Ficoll-Hypaque–purified positive T lymphocytes after gating. All cells outside "lymphocyte" light-scatter window were removed from analysis. Positive cells for each antigen are expressed as mean ± SD percentage of total cells analyzed (n = 10). Asterisk indicates $P<.01$; dagger, $P<.05$.

result, about 15% of all cells with lymphocyte morphologic features are excluded along with nonlymphoid cells. Significantly fewer cells ($P<.01$) fell within the lymphocyte gate in the preparations from infected animals than in burned or unburned control animals. Correspondingly, the number of cells with lymphocyte morphologic features was also decreased in the infected group.

The percentage of surface antigen–positive cells was recalculated for the cells falling within the lymphocyte window. The results of the recalculation are shown in Fig 3. Gating of the cells with higher-intensity scatter increased the proportion of W3/13- and W3/25-positive cells for both the burned and burned-infected groups. Gating also increased the proportion of W3/25-positive cells in the controls. There was no significant change in the proportion of OX-8–positive cells except for a decrease in the control group.

Although the percentage of positive cells for most antigens and the HSR changed after gating, a pairwise comparison of the groups using the gated data (Fig 4) illustrates that the relationships among the three groups remained relatively unchanged from the ungated analysis (Fig 1) for each of the lymphocyte subpopulations. Cells from burned-infected animals displayed the only significant changes from control: a decrease in the helper cells ($P<.01$), an increase in the suppressor cells ($P<.05$), and, as a result, a decreased HSR.

COMMENT

The changes in leukocyte morphologic features that occur after burn injury and burn injury with infection result in contamination of Ficoll-Hypaque–purified lymphocytes. This complex mixture of nonlymphoid cells could distort the values obtained for lymphocyte subpopulations in two ways. First, they could decrease the positive determinations by diluting the number of lymphocytes present in the preparation. Second, immature cells and nonlymphoid cells are often "sticky" and bind the fluorescent reagents in a nonspecific manner, and they might increase the observed percentage of positive values. Measures of in vitro lymphocyte function, such as phytohemagglutinin proliferation assays, could also be altered in an unpredictable manner by the presence of nonlymphoid cells. Consequently, lymphocyte subpopulation values and function tests determined on Ficoll-Hypaque–purified cell preparations from trauma patients need to be interpreted with caution.

Gating cells on a "normal" lymphocyte light-scatter window increased the percentage of positive values for both the pan-T and helper subsets in the burned and burned-infected groups. Gating also increased the percentage of cells positive for the helper subset in the control group. This increase in the proportion of positives after gating indicates that cells omitted by gating were negative for the subset marker used. These gated cells were monocytes in the control and burned animals but a more complex mixture of cells in the burned-infected animals. The fact that the suppressor subset was decreased by gating in the controls and unchanged in the experimental groups probably reflects OX-8 binding to some monocytes, a phenomenon that has been noted previously.[6]

The changes observed in percentage of positive values for the lymphocyte subsets with and without gating were not drastically different. The differences of the experimental groups from control were relatively unchanged by gating (Fig 1 vs Fig 4). Thus, although the results of the gated analysis were statistically different in most cases, the outcome of the experiment was not altered by gating.

Rats receiving a full-thickness burn covering 30% of total body surface area will normally survive if left unchallenged but are uniquely susceptible to infection. When given an infection challenge within 24 hours after injury, all will die with sepsis.[7] The burn injury–induced changes in host defense mechanisms responsible for this susceptibility are still unknown. Burn injury induces no significant change in the HSR in the infection-susceptible but uninfected animals, yet when this injury is complicated by infection we observe a drastically reduced HSR. Since the total lymphocyte count decreases, this change in ratio results from a greater depletion of helper cells than suppressor cells. In this burn infection model, changes in HSR were induced by infection rather than by burn injury, ie, with infection, more helper cells than suppressor cells disappear from the circulation.[8]

The HSR in peripheral blood has gained popularity as a measure of immunosuppression, yet the validity of subpopulation ratios as a measure of immunocompetence has been questioned.[9,10] Our test of the validity of the HSR as an indicator of immune capacity was to use a carefully controlled burn infection model[11] where infection susceptibility is clearly related to burn injury and the complexity of the peripheral leukocyte populations compared with those commonly induced in human patients by thermal injury is limited. We removed further ambiguity in the analysis by excluding cells with light-scatter intensity greater than that of lymphocytes. The clear dichotomy of the correlation between infection susceptibility induced by thermal injury and changes in the HSR in this model is further evidence that the HSR has limited applicability to prediction of infection susceptibility in burn victims.

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Discussion

DAVID AHRENHOLZ, MD, St Paul: Dr Burleson and his associates have critically looked at the lymphocyte populations isolated from rats by traditional Ficoll-Hypaque sedimentation techniques. From our facility, Dr Robert Nelson reported at the 1985 American Burn Association meeting in Orlando that some human neutrophils separated by Ficoll-Hypaque sedimentation layered out with the lymphocyte pool. We were concerned that in doing standard neutrophil function tests we were losing some neutrophil subpopulations during our separation technique, which were then not tested.

Dr Burleson and his associates have addressed the problem of whether contamination of these separated lymphocytes is affecting the reported levels of helper and suppressor T cells identified by monoclonal antibodies. They used flow cytometry with very narrow gate parameters to isolate pure lymphocytes at the expense of discarding some lymphocytic cells in the process. Significantly, the observed fall in T-cell numbers and the depression of the HSR remain in the burned and burned-infected animals even in the highly selected cell populations.

I have several questions for the authors. Based on this study, will they continue to use the Ficoll-Hypaque separation process or switch exclusively to the flow cytometric method? Have they tested these T cells identified by monoclonal antibody to see if they have retained their helper or suppressor function? Can they speculate on the reason for the loss of helper-cell activity in infected burned animals? In neutrophils we have observed that burn injury modulates expression of cell-surface antigens. Is the reduced helper-cell count a loss of monoclonal antibody–detectable surface receptors, a consumption of helper cells, or a failure of interleukin 2 to produce sufficient new helper cells in response to this marked stress?

J. JEREMY WOOD, BM, FRCS, Boston: I was responsible for writing up the data from our laboratory on studies instigated by Dr John O'Mahony, and they were published last year in the *Annals of Surgery.* Those, of course, were human studies. I would like to outline what our findings were, because they were not dissimilar from those of Dr Burleson and associates.

Essentially, in terms of methods, although we were using only four-directional scatter, we did use very tight lymphocyte gate settings, as the authors did, and we had similar findings, that if you had wide-open gates you saw lots of other cells that basically did not appear to be lymphocytes.

In terms of actual cells that we identified, we found that the T3 cells were reduced particularly if the burns were over 30% of body surface area, whereas there did not appear to be much effect due to sepsis on the distribution of T3 cells. However, when we looked at the T4 cells, the injury per se appeared to have no effect, whereas they were decreased in the group that was septic or subsequently became septic.

Have the authors looked at burns that are more extensive than the ones shown here, which I think were about 25% of body surface area? I believe they have experience with a larger burn in their model, and I think it would be interesting to know if there was any change in the T3 cells without a larger burn.

Finally, I also agree with the authors that the HSR is, by and large, not a very useful index, and if it is changed it is usually due to a decrease in T4 cells rather than an increase in T8 cells.

PAUL NATHAN, MD, Cincinnati: I wonder if the authors have considered that in gating the situation to the degree they did, they have eliminated a lot of cells. Obviously, they have selected out the normal lymphocytes and showed they are the same as in the normal animal. There are a lot of cells of unknown function in the group they are not measuring, which, let's say, are suppressor cells.

DR BURLESON: Dr Ahrenholz had several questions. As to whether we will continue to use Ficoll-Hypaque separation, because the leukocyte populations in burned patients are so complex, we have to concentrate the lymphocytes before analysis to obtain a sufficient number of lymphocytes to derive meaningful data. For the time being, we will continue to use Ficoll-Hypaque separations, but we are seeking to improve the flow cytometric techniques so that we can measure lymphocyte subpopulations in whole blood without preliminary purification.

In regard to the second question, we desire to use cell function in combination with cell phenotype as a measure of immunocompetence. Measurement of cell function may prove much more valuable than subpopulation analysis in evaluating host defense. We are investigating methods of measuring cell function by flow cytometry.

Third, as to the reason for the reduced helper count, we do not have complete data on this, but my own bias is that compartmentalization is occurring. You can induce similar kinds of changes by administering steroids. For instance, administration of hydrocortisone can temporarily reduce lymphocyte counts and shift the lymphocyte subpopulations, apparently through forcing the lymphocytes into systemic compartments for short periods.

Dr Wood asked whether we have looked at more extensive burns. We have done some preliminary work with larger burns, and I can say the cell populations are even more complex than in the 30% burns. The question we wanted to answer in this study, though, was whether a 30% burn, which induces infection susceptibility, correlates with a shift in subpopulations. We chose not to confuse that issue by using a larger burn with more complex cell compositions.

In answer to Dr Nathan's question about the morphologic features of the gated cells, we are planning to sort the gated populations and determine the morphologic characteristics of the cells that are removed by light-scatter gating. In other words, we will sort out the cells based on light scatter and study the cells with a particular light-scatter profile by light microscopy after Wright's stain.

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